

Kombocles bakaiana gen. sp. nov. (*Boletaceae*), a new sequestrate fungus from Cameroon*

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Abstract: *Kombocles bakaiana* gen. sp. nov. is described as new to science. This sequestrate, partially hypogeous fungus was collected around and within the stilt root system of an ectomycorrhizal (ECM) tree of the genus *Uapaca* (*Phyllanthaceae*) in a Guineo-Congolian mixed tropical rainforest in Cameroon. Molecular data place this fungus in *Boletaceae* (*Boletales*, *Agaricomycetes*, *Basidiomycota*) with no clear relationship to previously described taxa within the family. Macro- and micromorphological characters, habitat, and DNA sequence data are provided. Unique morphological features and a molecular phylogenetic analysis of 304 sequences across the *Boletales* justify the recognition of the new taxa. *Kombocles bakaiana* is the fourth sequestrate *Boletaceae* described from the greater African tropics, and the first to be described from Cameroon.

Key words:

ectomycorrhizas
false truffle
Guineo-Congolian rainforest
hypogeous fungi
Uapaca

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INTRODUCTION

Numerous genera of sequestrate fungi within *Boletaceae* (*Boletales*) have been recognized from various regions of the world, including the widely distributed north temperate *Chamonixia*, *Gastroboletus*, and *Octaviania*, Australasian *Rossbeevera*, South-East Asian *Durianella*, *Spongiforma*, and *Rhodactina*, Australian *Soliococcus* and *Royoungia*, and tropical South American *Castellanea*, *Costatisporus*, and *Jimtrappea* (Binder & Bresinski 2002, Desjardin *et al.* 2008, 2009, Halling *et al.* 2012, Lebel *et al.* 2012, Orihara *et al.* 2012a, b, Moreau *et al.* 2013, Trappe *et al.* 2013, Smith *et al.* 2015). For the African tropics, despite a high diversity of non-sequestrate, epigeous *Boletaceae* known from some areas (e.g. Heinemann & Goossens-Fontana 1954), known sequestrate *Boletaceae* are currently limited to a single species each of *Mackintoshia*, *Mycoamaranthus*, and *Octaviania* (Dissing & Lange 1962, Castellano *et al.* 2000, Mueller *et al.* 2007, Pacioni & Sharp 2000, Tedersoo & Smith 2013). Of late, the sequestrate *Lactarius megalopterus* (*Russulaceae*) was described from lowland rainforests of Cameroon (Beenken *et*

al. 2016). Our recent collecting in the Dja Biosphere Reserve of southern Cameroon discovered a number of sequestrate fungi, including the first continental African records for *Elaphomyces* ascomata from native plant communities (Castellano *et al.* 2016). Included in the Dja Biosphere Reserve collections is a morphologically distinctive fungus that produces basidiomata gregariously in soil in lowland, mixed tropical rainforests in close proximity to ectomycorrhizal (ECM) trees of the genus *Uapaca* (*Phyllanthaceae*). Molecular and morphological data indicate that this Cameroonian sequestrate fungus is a member of *Boletaceae* but is evolutionarily distinct from all other described epigeous and sequestrate genera and species within the family.

MATERIALS AND METHODS

Collections

Basidiomata were collected during the August–September early rainy season of 2014 from Cameroon's Dja Biosphere Reserve, Northwest Sector near the village of Somalomo, Upper Dja River Basin, ~1.4 km west of a base camp located at 3°21'29.8" N 12°43'46.9" W, 650 m a.s.l., in mixed forest containing *Uapaca* species (Peh *et al.* 2014).

Descriptions of macromorphological features are from fresh material in the field. Colour terms follow Kornerup &

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Wanschler (1978) and are cited in parentheses (e.g. 5A4). Collections were dried with silica gel. Preserved specimens were later examined in 3 % KOH, Melzer's reagent, and Cotton blue. Microscopic descriptions are based on 3 % KOH mounts unless specified otherwise. Twenty basidiospores were measured from the type collection. Dried basidiospores were mounted on aluminum pegs with double-sided tape and coated with gold for scanning electron microscopy (SEM) with an AmRay 3300 FE field emission scanning electron microscope using 5 kV. Type specimens are deposited in the following herbaria: YA (Cameroon National Herbarium, Yaoundé); HSC (Humboldt State University, Arcata, CA); OSC (Oregon State University, Corvallis); K(M) (Fungarium, Royal Botanic Gardens, Kew).

DNA extraction, PCR amplification, and sequencing

All DNA work was carried out in the Jodrell Laboratory, Royal Botanic Gardens, Kew. DNA extractions were performed on dried basidioma tissue using the Extract-N-Amp Plant PCR kit (SIGMA-ALDRICH, Saint Louis, MO), followed or not by plate filtration (Dentinger *et al.* 2010), or using a Plant DNeasy mini kit (QIAGEN, Valencia, CA). Full ITS 1 and 2 regions, along with the 5.8S rDNA (ITS), were PCR-amplified with primers ITS1F and ITS4 (White *et al.* 1990, Gardes & Bruns 1993), and the nuclear 28S rDNA D1–D2 domains (28S) were PCR-amplified with LR0R/LR5 (Vilgalys & Hester 1990) following the cycling conditions in Dentinger *et al.* (2010). PCR products were visualized by UV-fluorescence after running out 2 µL PCR products in a 1 % agarose gel containing 0.005 % ethidium bromide. Prior to sequencing, amplicons were cleaned of unincorporated dNTPs and excess primers by adding 1 µL ExoSAP-IT (USB, Cleveland, OH) to 5 µL PCR reaction mix and incubating for 15 min at 37 °C followed by 15 min at 80 °C. Unidirectional dye-terminator sequencing used BigDye3.1 (Applied Biosystems, Foster City, CA) by adding 2 µL of cleaned PCR template to 3 µL of solution containing 0.2 µL BigDye, 1 µL sequencing buffer, 0.15 µL 50mM MgCl₂, 0.15 µL of 10 µM primer, and 1.5 µL of Milli-Q (Merck Millipore, Darmstadt, Germany) purified water. Sequencing was performed with 60 cycles of 95 °C denaturation for 10 sec, 50 °C annealing for 10 sec, and 60 °C extension for 2 min. Sequencing reactions were cleaned using ethanol precipitation and resuspended in purified water before loading into an ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA). Complementary unidirectional sequence reads were aligned and edited in Sequencher v. 4.2 (Gene Codes, Ann Arbor, MI) and deposited in GenBank (ITS: KX827004; 28S: KX827003).

Taxa used, sequence alignment, and phylogenetic analysis

The ITS ribosomal DNA sequence from the new taxon was initially subjected to a BLASTn query against GenBank in order to explore its putative phylogenetic relationships. To further assess phylogenetic affinities of the new fungus, we used Maximum Likelihood (ML) analysis of an ultra-large alignment of all ITS sequences from the UNITE general release v. 7 (release date 31 Jan. 2016) (Nilsson *et al.* 2015) classified as *Boletaceae* and identified as full-length by ITSx (Bengtsson-Palme *et al.* 2013), excluding two misclassified

sequences. The final ITS dataset consisted of 717 sequences. We also used ML analysis of a large dataset based on 28S sequences of diverse *Boletaceae* with additional *Boletales* taxa as outgroups. The 28S analysis included our original sequence data, 288 sequences used in Wu *et al.* (2014), seven sequences from sequestrate genera used in Smith *et al.* (2015), and eight sequences from the *Heimioporus* species used in Halling *et al.* (2015). The final dataset consisted of 304 sequences representing species from infrafamilial clades across the *Boletaceae* based on recent studies (e.g. Nuhn *et al.* 2013, Wu *et al.* 2014, 2015, Smith *et al.* 2015, Henkel *et al.* 2016) and outgroup taxa. Representatives from all available sequestrate *Boletaceae* genera were included: *Castellanea*, *Chamonixia*, *Costatisporus*, *Durianella*, *Gymnogaster*, *Heliogaster*, *Jimtrappea*, *Mackintoshia*, *Mycoamaranthus*, *Neoboletus*, *Octaviana*, *Rossbeevera*, *Royoungia*, *Soliococcus*, and *Spongiforma*.

Each dataset was aligned separately using default settings in PASTA (Mirarab *et al.* 2015). For both datasets, phylogenetic analysis under the ML criterion was performed using the Pthreads parallelised version of RAXML v. 8.2.4 (ITS) or v8.2.9 (28S) (Stamatakis 2006, Ott *et al.* 2007) with a GTRGAMMA model, allowing model parameters to be estimated for each gene partition separately. Branch support was assessed using nonparametric bootstrapping with the autoMRE option. The alignments and trees have been deposited in TreeBASE and are available at <http://purl.org/phylo/treebase/phyloids/study/TB2:S19818>.

RESULTS

BLASTn queries and phylogenetic analysis

Using GenBank BLASTn queries of the new taxon's ITS sequence, the top 100 best hits all belonged to *Boletaceae* but were uninformative at the generic level. The sequence from the new taxon was, however, nearly identical (99 % over 606 nucleotides) to a sequence (FR731937) from an ECM root tip of *Uapaca guineensis* from Gabon sampled by Tedersoo *et al.* (2011). The BLASTn matches to remaining sequences in GenBank covered only a maximum of 79 % of the query sequence with 86 % identity. Final alignments for phylogenetic analysis consisted of 717 sequences and 4009 aligned positions for the ITS (1476 parsimony informative, 2119 constant, 414 autapomorphic), and of 304 sequences and 1293 positions for the 28S (441 parsimony informative, 634 constant, 140 autapomorphic). All characters were included in the analysis of both datasets. For the ITS dataset, RAXML rapid bootstrapping terminated after 350 replicates (WRF average of 100 random splits = 2.603297) and the best ML tree had a likelihood score of -60202.046042. For the 28S dataset, RAXML rapid bootstrapping terminated after 300 replicates (WRF average of 100 random splits = 2.696777) and the best ML tree had a likelihood score of -31601.477718 (Fig. 1). A second analysis of the 28S dataset after removing ambiguously aligned regions in GBLOCKS (Castresana 2000) with the option to allow gap positions in the final blocks (468 positions retained) failed to recover a monophyletic *Boletaceae*, evidence that phylogenetically informative regions were lost (data not shown). In both the ITS

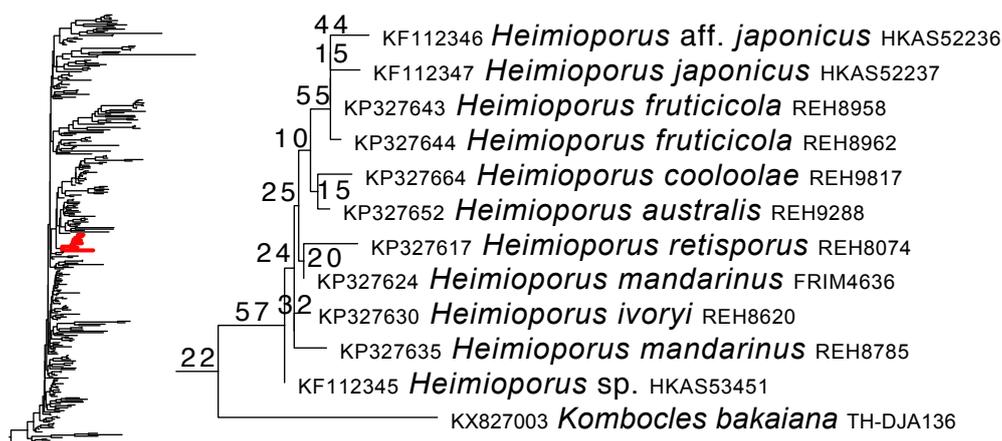


Fig. 1. Phylogenetic placement of *Kombocles bakaiana* within *Boletaceae* using Maximum Likelihood (ML) analysis of 28S ribosomal gene sequences. Left is the best ML tree from a RAxML search using the GTRGAMMA model of substitution on a PASTA-aligned dataset of 288 *Boletaceae* sequences and 16 outgroup taxa from other *Boletales*. The tree is rooted at the node leading to *Suillus* spp. The putative clade containing *Kombocles bakaiana* is highlighted in red and shown on the right as an enlargement. Numbers on branches are percentage support based on 300 nonparametric bootstrap replicates. Terminal labels are composed of the GenBank accession number followed by taxon name and collection code.

and 28S datasets, the new Cameroonian taxon was placed within *Boletaceae*, but was not nested within, or supported as related to, any previously described epigeous or sequestrate genera. Although the 28S analysis recovered the new taxon in a clade with the epigeous bolete genus *Heimioporus*, this putative relationship had very low bootstrap support (22 %; Fig. 1), as did other nodes deeper than the terminal clades in the tree.

TAXONOMY

Kombocles Castellano, T.W. Henkel & Dentinger, *gen. nov.*

MycoBank MB818594
(Fig. 2)

Etymology: *Komba* and *-cles* (Gk. suffix m), “Komba the renowned”, referring to the spiritual protector and provider of the forest as recognized by the Baka indigenous people of the type locality of the Dja Biosphere Reserve.

Diagnosis: Distinguished from other *Boletaceae* by a combination of the following characters: *Basidiomata* emergent, sequestrate, globose to subglobose. *Peridium* various shades of brown, unchanging. *Gleba* brown with irregular off-white veins, unchanging, loculate. *Columella* absent. *Basidiospores* asymmetrical, variously shaped from fusoid to allantoid to unevenly ellipsoid, rugulose, yellow-brown, dextrinoid when young, with a distinct hilar appendage. *Cystidia* and clamp connections absent.

Type species: *Kombocles bakaiana* Castellano *et al.* 2016.

Kombocles bakaiana Castellano, T.W. Henkel & Dentinger, *sp. nov.*

MycoBank MB818595
(Fig. 2)

Etymology: *Baka* and *-iana* (L. suffix adj.), i.e. “of the Baka”; referring to the Baka indigenous people inhabiting the type locality.

Diagnosis: Differs from other known sequestrate taxa by the combination of the pale cream to light yellowish brown peridium, gleba with brown locules unevenly surrounded by bright white to off-white tramal tissue, lack of a columella, yellow-brown basidiospores that appear smooth under light microscopy but rugulose under SEM, are dextrinoid when young with a distinct hilar appendage, uniquely long sterigmata, and absence of cystidia.

Type: **Cameroon:** *East Province:* Dja Biosphere Reserve, Northwest Sector near the village of Somalomo, Upper Dja River Basin, ~1.4 km west of Dja base camp located at 3°21'29.8" N 12°43'46.9" W, in mixed forest, around and in between the stilt roots of *Uapaca* sp., near *Gilbertiodendron dewevrei* monodominant plot 3, 25 Sept. 2014, *Henkel THDJA 136* (YA 66911 – holotype; HSC G1203, OSC 150017, K(M) 205361 – isotypes.) GenBank accession numbers ITS: KX827004; 28S: KX827003.

Description: *Basidiomata* irregularly globose to subglobose, 10–20 mm tall, 15–25 mm broad, firm throughout development; surface pale cream to light yellow brown (4A2–5C4), inherently smooth but with numerous adherent soil particles, occasionally with irregularly spaced cracks revealing the underlying gleba, unchanging with handling but drying rugose to ruminant with numerous shades of brown and darker, humic-stained patches. *Peridium* two-layered in cross-section; outer layer < 0.5 mm thick, concolorous with the surface but somewhat mottled due to uneven distribution of humic stains, underlain by an inner, pale yellowish (1A2–1A3) layer up to 1 mm thick that becomes cottony near the gleba. *Gleba* initially marbled, then with increasingly well-defined locules separated by off-white to bright white veins, with clear exudate centrally when cut in mature specimens;

locules initially off-white to yellowish orange (4B7) to brown (7E8–7F8) at maturity; locules nearly filled with hymenial elements and basidiospores, irregularly spherical, up to ± 1 mm broad. *Dried gleba* distinctly loculate; locules circular to irregularly polygonal, variably-sized up to 1.1 mm broad, with a dark, thin (± 20 μm) outline, inner portion mottled yellow-brown to medium brown, often with a paler central core, contiguous to irregularly-spaced. *Odour* mild, pleasant, somewhat sweet. *Taste* mild, indistinct.

Peridium 220–320 μm thick, two-layered; outer layer ± 20 μm thick but non-uniform in thickness and sometimes absent in small portions of section, of pale yellow-brown tissues that are distinctly and heavily encrusted with minute particles of unknown origin, otherwise of similar structure as underlying inner layer; inner layer 200–300 μm thick, structurally a mix of *textura globosa* and *textura intricata* appearing as interwoven tissue with abundant hyaline, elongate, clavate cells, these 26–31 \times 13–16(–24) μm , smaller towards peridial surface and larger where the layer adjoins the gleba; walls up to 2 μm thick. *Glebal trama* similar in structure to the inner peridium but with less elongate hyphae and more clavate or irregularly-shaped cells, variable in thickness to nearly absent, with no apparent color differentiation near the outer locule edge. *Basidia* elongate, 40–45 \times 6–7 μm , hyaline, not rehydrating well; sterigmata four, prominent, 4–8.5 \times ± 1 μm , hyaline. *Basidiospores* variably-shaped, fusoid, allantoid to unevenly ellipsoid, asymmetrical in side view, with slight suprahilar depression, smooth under bright field microscopy or faintly roughened with Nomarski imaging, but distinctly rugulose under SEM, in KOH yellow-brown singly and in mass, many distinctly and instantly dextrinoid, particularly those towards the center of some locules, (12.5–)13.5–16.5 \times (5–)5.5–6.0 μm (mean 14.3 \times 5.7 μm), Q mean = 2.50, range = 1.86–2.67(–3.17) excluding the hilar appendage; hilar appendage prominent, up to 1.5 μm long, hyaline; walls 1.0–1.5 μm thick.

Habit, habitat, and distribution: Scattered and subhypogeous in leaf litter within and around the stilt-root system of an ectomycorrhizal (ECM) *Uapaca* sp. in mixed forest; known only from the type locality in the Dja River Basin of southern Cameroon. A nearly identical ITS sequence originating from an ECM root tip of *U. guineensis* from Gabon suggests that the fungus may have a wider distribution in the Guineo-Congolian rainforest.

Commentary: *Kombocles bakaiana* is recognized in the field by its subglobose basidiomata that remain firm to maturity, pale cream to light yellowish brown peridium, gleba with well-defined locules that are initially white, maturing to yellowish orange and eventually to brown, lack of a columella, and mild odour and taste. Currently basidiomata of *K. bakaiana* are only known in close spatial association with *Uapaca* trees in forest stands lacking ECM *Gilbertiodendron dewevrei* trees, and a conspecific ITS sequence has been recovered from a *Uapaca* root tip from Gabon (Tedersoo et al. 2011), so it may be presumed that *Uapaca* is the ECM host. Micromorphologically, *K. bakaiana* is distinguished by the variably shaped, asymmetrical, yellow-brown, dextrinoid, smooth basidiospores with a distinct hilar appendage. In the

phylogenetic analysis, *K. bakaiana* was putatively sister to the Australasian epigeous bolete genus *Heimioporus* albeit with very low bootstrap support (Fig. 1). While obviously different macromorphologically (epigeous and non-sequestrate in *Heimioporus*; hypogeous and fully sequestrate in *Kombocles*), the basidiospores of *H. cooloolae* have a somewhat similar shape to those of *K. bakaiana* and are also dextrinoid (though rarely), but differ in having a pitted surface ornamentation compared to the non-pitted basidiospore surface of *K. bakaiana* (Halling & Fechner 2011, Halling et al. 2015).

Macromorphologically, *K. bakaiana* is reminiscent of species of *Melanogaster* and *Alpova* (*Paxillaceae*), but the dextrinoid, large, yellow-brown basidiospores allow easy separation from these two genera (Moreau et al. 2011). Microscopically *K. bakaiana* basidiospores are similar to the smooth, subfusiform basidiospores of *Castellanea* and *Jimtrappea* (*Boletaceae*) from tropical forests in Guyana, but these two genera have symmetrical, pedicellate basidiospores compared to those of *K. bakaiana* that have a distinct hilar appendage and are variably-shaped and asymmetrical (Smith et al. 2015). In addition, *Jimtrappea* has dextrinoid hymenial cystidia, which are absent in *K. bakaiana*. *Soliococcus* (*Boletaceae*) from Queensland, Australia, has variably shaped, pale yellow basidiospores that are nondextrinoid (Trappe et al. 2008).

Among the few sequestrate putatively ECM fungi known from the African tropics, *Mackintoshia persica* from Zimbabwe is characterized by smooth, dextrinoid, thick-walled basidiospores that are very similar to those of *K. bakaiana* (Castellano et al. 2000, Pacioni & Sharp 2000). *Mackintoshia persica* was initially assigned to *Cortinariaceae* (Pacioni & Sharp 2000) but recent analyses have revealed that it is a member of *Boletaceae* (Smith et al. 2015). In contrast to *K. bakaiana*, basidia of *M. persica* lack distinct sterigmata, hymenial cystidia are present, and the basidiospores are uniformly ellipsoid to broadly ellipsoid. *Mycoamaranthus congolensis*, known from the Congo, Malawi, and Zimbabwe, while confirmed recently as belonging to *Boletaceae* (Smith et al. 2015), differs from *K. bakaiana* primarily in the spinose basidiospore ornamentation (Castellano et al. 2000). *Octaviania ivoryana*, while widespread in Africa, being known from Guinea, Kenya, Senegal, and Zimbabwe, differs fundamentally from *K. bakaiana* in the cone-like ornamentation of its basidiospores (Castellano et al. 2000). While the phylogenetic position of *O. ivoryana* is currently unknown, other members of the genus are members of *Boletaceae* (Smith et al. 2015, Orihara et al. 2012a). The sequestrate *Corditubera staudtii* was originally described from Cameroon, but differs from *K. bakaiana* in the reddish (vs. brown loculate in *K. bakaiana*) gleba and basidiospores that are globose with spinose ornamentation (Hennings 1897).

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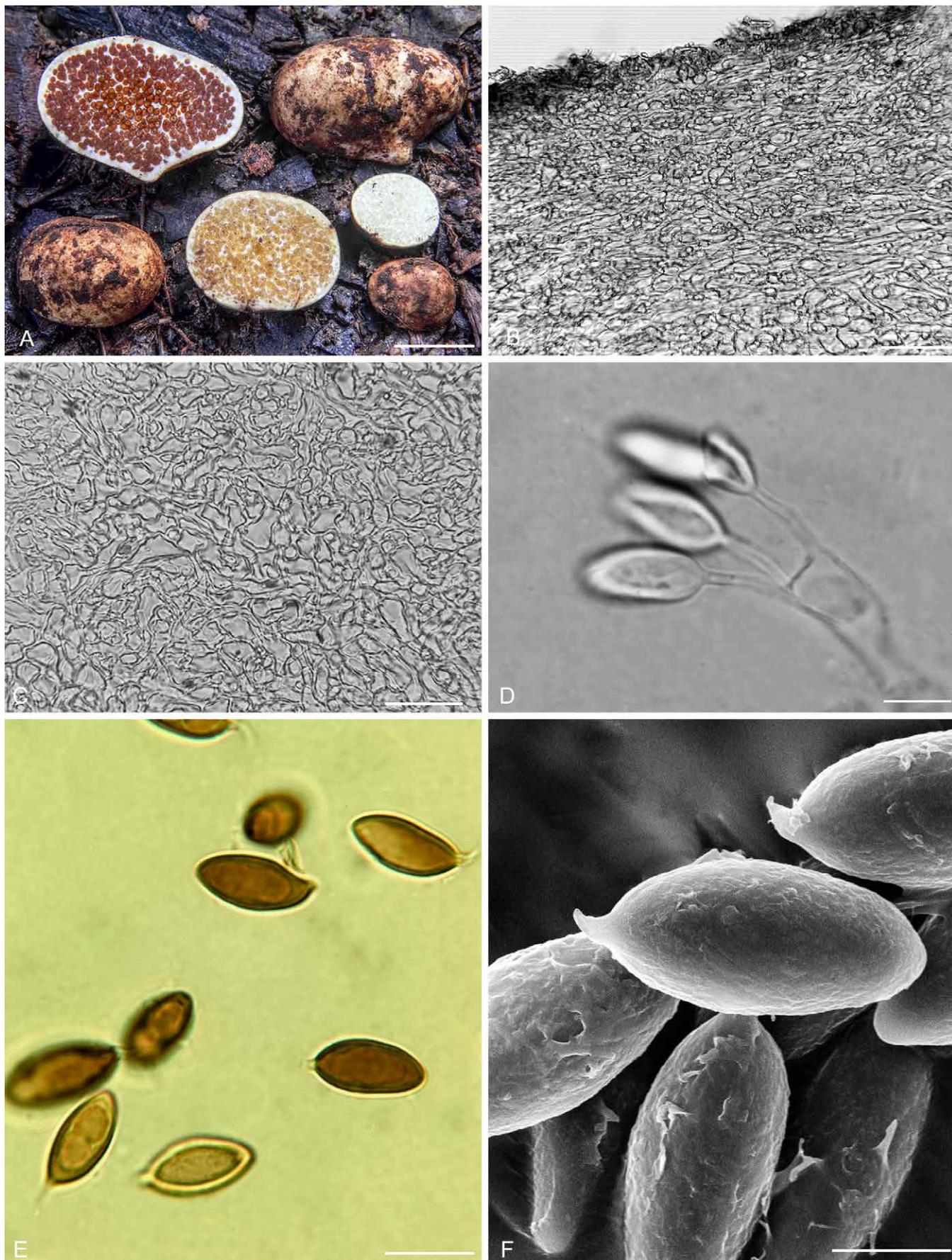


Fig. 2. *Kombocles bakaiana* (YA 66911 – holotype;). **A.** Basidiomata in section showing peridial surface, peridium, and gleba. **B.** Cross-section of outer (i.e. the thin layer of dark cells) and inner peridium. **C.** Cross-section showing tramal structure. **D.** Basidium with attached basidiospores and long sterigmata. **E.** Basidiospores in surface and cross-sectional view. **F.** Scanning electron micrograph of basidiospores showing the fine detail of the ornamentation. Bars A = 10 mm, B = 50 μ m, C = 25 μ m, D = 6 μ m, E = 10 μ m, and F = 5 μ m.

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